Determination of Sclerotial Populations of Sclerotium rolfsii in Soil by a Rapid Flotation-Sieving Technique

R. Rodriguez-Kabana, P. A. Backman, and Elizabeth A. Wiggins

Respectively, Alumni Associate Professor, Assistant Professor, and Graduate Assistant, Department of Botany and Microbiology, Agricultural Experiment Station, Auburn University, Auburn, Alabama 36830.

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ABSTRACT

A flotation technique using an aqueous solution of blackstrap molasses, was developed for rapid extraction of sclerotia of *Sclerotium rolfsii* from soil. A 50-ml (70 g) subsample of air-dried, screened (4-mm mesh) soil was placed in a 600-ml beaker and enough extracting solution added to provide a final volume of 350 ml. The mixture was stirred with an electric stirrer (1,600 rpm) for 30 s and allowed to settle for 30 s. The liquid was decanted through a 250 μm (60-mesh) screen of 7.5 cm diam, and the sclerotia were counted and plated on selective oxalate-gallic acid medium to determine viability. The extracting solution consisted of 200 ml blackstrap molasses (sp gr 1.300, 25 C), 800 ml tap water, and 12.5 μg/ml Separan NP 10 (a flocculating agent). This extracting solution had a sp gr value of 1.073 (25 C), and was equivalent in extracting efficiency to a 0.75 M sucrose solution. The recovery of sclerotia using this technique was greater than 80% of those present in a soil sample and was sensitive to a concn as low as one sclerotium per 50 ml soil. When used to study a 2-yr peanut-corn rotation, the technique revealed no sclerotia in soil from fields under corn which had been under peanuts the previous year. An average of 3.33 sclerotia/250 ml soil was found in fields under peanuts which had been under corn the previous year. Results of studies on the distribution of sclerotia in two fields under peanut monoculture indicated a heterogenous distribution of sclerotia and a pronounced "row effect" where plots were contiguous.

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Additional key words: extraction method, ecology of sclerotia.

Studies of dissemination, survival, and general ecology of *Sclerotium rolfsii* Sacc. in the field require a rapid, economical, and simple procedure for the extraction of sclerotia from soil. The method should be capable of handling the great number of samples required in "mapping" fairly large areas. While the standard procedure of repeated washing and sieving of soil (7) is very effective, it requires considerable time (10-15 min) for each determination. It was thought that a possible answer to these problems could be an adaptation of the flotation-sieving technique used for the extraction of nematodes from soil (3) to the extraction of sclerotia. The method would be based on the use of an extraction solution containing a flocculating agent and a specific gravity equal to or higher than that of the sclerotia.

The objectives of this study were: (i) to describe a rapid technique we have developed for the extraction of sclerotia of *S. rolfsii* from soil; (ii) to discuss conditions and limitations of the method; and (iii) to show field application of the method in determining survival and

general distribution of sclerotia in fields under peanut monoculture and peanut-corn rotation.

MATERIALS AND METHODS.—*Extraction procedure.*—The basic procedure used to extract sclerotia from soil consisted of the following steps: Soil samples were air-dried and screened through a coarse (4 mm) sieve. A 50 ml subsample was placed in a 600-ml beaker and enough extracting solution was added to have a final volume of 350 ml. The mixture was stirred with a propeller type stirrer (1,600 rpm) for 30 s and allowed to settle for 30 s. The liquid was then rapidly decanted through a 250 μm (60 mesh) screen 7.5 cm in diam. Material collected was washed into a plastic dish with three concentric ridges 1 cm apart and sclerotia in the dish were counted under a diffuse white light.

The extracting solution consisted of 200 ml blackstrap molasses (sp gr 1.300, 25 C) 800 ml tap water, and 12.5 μg/ml of the flocculating agent Separan NP 10 (The Dow Chemical Co., Midland, Mich.). This extracting solution had a sp. gr. of 1.073 (25 C).

Determinations of specific gravity were made at 25 C with standard hydrometers.

Viability of sclerotia was determined on filter-sterilized oxalate-gallic acid medium (2) containing (g/liter): potassium oxalate ($K_2C_2O_4$), 10; KH_2PO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.5; KNO_3, 2; and thiamine hydrochloride, 0.001. The medium was adjusted to a pH of 4.2 and provided with 10 ml of a minor element solution containing (g/liter): $FeSO_4 \cdot 7H_2O$, 1; $MnSO_4 \cdot H_2O$, 0.6; and $ZnSO_4 \cdot 7H_2O$, 1. Plates of sclerotia were incubated overnight at 27 C then covered with a layer of 0.01 N iodine solution for 30-40 s until the surface of the medium turned black. The plates were transferred to a shallow pan containing warm water (35-40 C) and the iodine washed off until a halo appeared around living sclerotia; dead sclerotia lacked this property.

Recovery of sclerotia in relation to specific gravity.—The recovery of sclerotia in relation to specific gravity of the extracting medium was determined using solutions of varying specific gravity. Solutions with sp gr values lower than 1.000 were prepared by mixing 95% (v/v) ethanol with water; solutions with sp gr greater than 1.000 were aqueous sucrose solutions of increasing molarity. Specific gravities used were over the range of 0.785 to 1.173.

Sclerotia for this study were collected from 4-wk-old cultures of *S. rolfsii* in sterilized soil amended with 1% (w/w) cornmeal. To determine the percentage of sclerotia that floated in each solution, 200-400 sclerotia were placed in a 100-ml beaker and 40-50 ml of the solution was added. The mixture was then vigorously shaken and allowed to stand for 1 min; contents of the beaker were then swirled and poured into a 90-mm diam petri dish. Sclerotia left in the beaker were washed into the dish with an additional 20-30 ml of the solution. The number of sclerotia floating was counted, and the percent of the total that floated was calculated. Each determination was performed in triplicate using different sclerotia each time.

Relation between specific gravity and the number of sclerotia extracted from soil.—This relation was established by use of a series of solutions with increasing specific gravities prepared by dissolving various quantities of blackstrap molasses in tap water. The range of specific gravity covered in this study was from 1.000 to 1.151. Sclerotium-infested soil for this experiment was obtained from pure cultures of *S. rolfsii* in a soil-cornmeal medium. Five replications were performed for each solution following the standard extraction procedure.

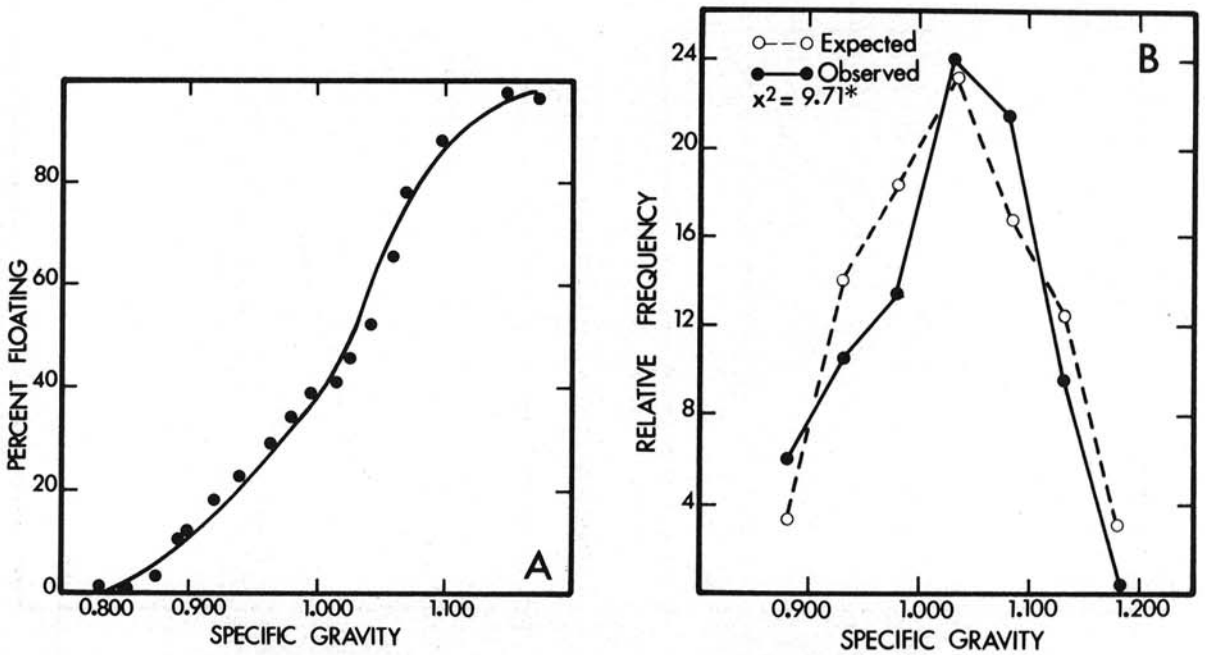

Fig. 1-(A, B). Relation between specific gravity of solutions and the number of sclerotia floating: **A)** Cumulative plot; **B)** Predicted and observed percent frequency distribution of floating sclerotia with respect to sp gr. (*indicates significant chi-square values, $P = 0.05$.).

Volume of extracting solution and size of soil sample.—Two experiments were conducted to determine the flexibility of the extraction procedure with regard to amount of molasses solution and size of soil sample processed. The soil used was collected from a field under continuous peanut culture. In one experiment, 50-cc samples of soil were processed using increasing amounts of molasses solution to give final volumes in 350, 450, 550, and 650 ml. Ten replications were prepared for each final volume of extracting solution.

Two soil sample sizes, 50 and 100 cc, were tested in a fixed final volume of 350 ml with molasses solution. Each sample size was represented by 11 determinations.

Extraction efficiency.—The effectiveness of the extraction procedure for removal of sclerotia from soil was tested by adding sclerotia to 50-ml samples of cornfield soil containing no sclerotia. Sclerotia for this study were collected in a peanut field and were added at levels of 1 and 10 per 50 ml of soil. These levels represented the range of concn of sclerotia found in peanut fields. Six determinations for each level were performed using both water and molasses solution.

Replications.—The number of determinations (n) required per soil sample was studied in relation to various levels of allowable error (L) using the formula:

$$n = \frac{4\ SD^2}{L^2}$$

where SD represents the standard deviation (8). Data for determination of SD were obtained from 24 replicate extractions of soil from a peanut field. Various values for n were then calculated based on the SD value obtained by considering L values from 1-10 sclerotia per 50 ml of soil.

Field studies.—The extraction procedure was applied in the determination of numbers of sclerotia in soil collected in Dec. 1972 from plots of a study on peanut-corn rotation. Soil cores 2.5 cm in diam were collected to a depth of 15 cm along the center portion of 3.65 × 15.25-m plots. Composites of 20-25 cores from each plot were pooled, mixed well, and air-dried at room temp (25-28 C). Samples thus collected were obtained from eight plots which were under peanut culture in 1972 and corn in 1971. An equal number of plots that were under corn in 1972 and peanuts in 1971 were also sampled.

The distribution of sclerotia in soils under peanut monoculture was studied in two fields that had been under peanut culture for at least 4 yr. Soil samples (15-20 cores per plot) were collected in Dec. 1972 from 3.65 × 9.15-m plots as described above. A total of 102 plots were sampled in the two fields.

Samples from all field studies were processed to determine sclerotia according to the extraction procedure described in this paper and a total of five determinations were performed per sample.

Statistical analyses.—Results obtained from the study of the frequency distribution of sclerotia in relation to sp gr were analyzed for conformance with the normal curve model using procedures described by Snedecor (8). All data were statistically analyzed following standard procedures for analysis of variance. Unless otherwise indicated, all differences referred to in the text were significant at the 95% level of probability.

RESULTS.—*Frequency distribution of sclerotia in*

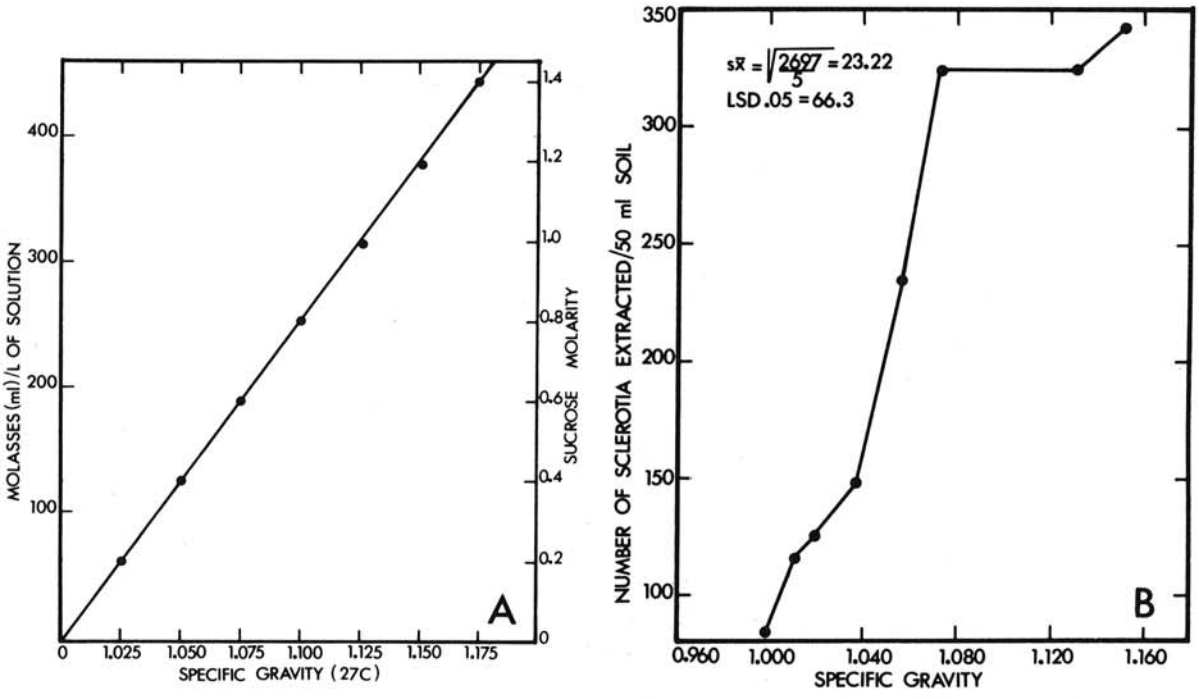

Fig. 2-(A, B). Extraction of sclerotia from soil using molasses solutions: **A)** Relation between sp gr, sucrose molarity, and volume of blackstrap molasses per liter of solution; **B)** Relation between sclerotia extracted and sp gr (25 C) value of the extracting solution.

relation to specific gravity.—Changes in percentage of sclerotia that floated in extracting solutions of increasing sp gr values are represented in Fig. 1-A. The results indicated that no sclerotia floated in solutions with sp gr values of 0.819 or less. The percentage of sclerotia floating increased slowly with increasing sp gr from 0.819 to 0.900; the response to increasing sp gr within the range of 0.900 to 1.100 was sharp and positive. The greatest increase in percent of sclerotia floating occurred between sp gr values of 1.030 and 1.073. Above 1.073, the percent of sclerotia floating changed at a progressively slower rate, until little change was observed between sp gr values of 1.149 and 1.173.

The sigmoid shape of the curve in Fig. 1-A can be considered as equivalent to a cumulative frequency plot of the normal or Gaussian curve. A test of conformance to this model was made from an increment plot of data in Fig. 1-A. The plot was based on increments in sp gr equal to 0.050 and considered the percentage value corresponding to a sp gr value of 1.030 as the inflection point; i.e., the mean for the curve in Fig. 1-A. Figure 1-B shows the theoretical values which would be anticipated for each sp gr increments of 0.050 together with the observed values. The chi-square test for significance indicated that the normal model provided a good description ($\chi^2 = 9.71$*) of the relation between percent floating sclerotia and changes in sp gr of solutions. The theoretical distribution of sclerotia indicated that 95% of sclerotia have specific gravities within the range of 0.932 to 1.191.

Number of sclerotia extracted from colonized soil in relation to specific gravity.—The relation existing between sucrose molarity, the volume of blackstrap molasses in extracting solutions, and the sp gr is presented in Fig. 2-A. This relation was used to express data obtained from the frequency distribution studies with sucrose solutions in equivalent terms of the more economical molasses solutions. Numbers of sclerotia removed from soil (Fig. 2-B) increased with increasing sp gr of extracting molasses solutions in the range 1.000 to 1.072; increasing sp gr values of the solution above 1.072 did not result in any significant increase in the number of sclerotia extracted.

Effect of extracting solution volume and soil sample size.—Changes in final volume in the mixing beaker from 350 to 550 ml, resulted in no significant change in the number of sclerotia extracted from 50 ml of soil. With a total volume of 650 ml there was a significant decline in

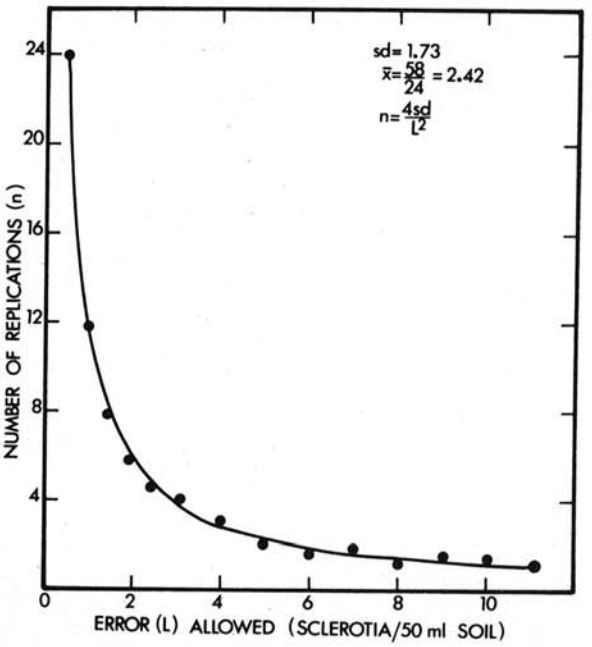

Fig. 3. Relation between the number of replications and the error allowed in order to obtain differences significant at the 5% level of probability.

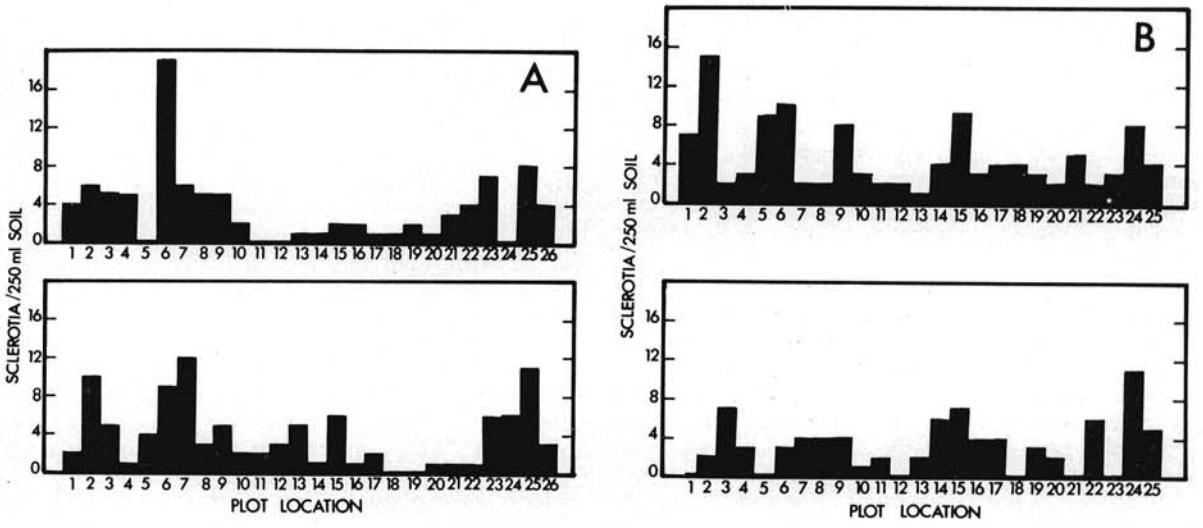

Fig. 4-(A, B). Distribution of sclerotia in two fields under peanut monoculture: **A)** plots in contiguous tiers; **B)** plots in two tiers separated by a 6-m-wide uncropped alley.

the number of sclerotia extracted. Doubling the size of soil samples from 50 to 100 ml (while maintaining a final volume of 350 ml in the mixing beaker) resulted in an increase of extracted sclerotia from 4.5 to 6.5 in an average of 11 determinations.

Efficiency of extraction.—The molasses solution (sp gr 1.073, 25 C) was superior to water for the extraction of field sclerotia added to soil. At a level of one sclerotium per 50 ml of soil, the molasses solution was successful in 83% of the trials in removing the added sclerotium; water was only successful in 33% of the trials. The corresponding percentages of success for the level of 10 sclerotia per 50 ml of soil were 81.6 for molasses and 45% for water.

Replications.—The average number of sclerotia extracted in 24 determinations from a peanut soil was 2.42 and the SD was equal to 1.73. Figure 3 illustrates the relation between the number of replications and error allowed. Calculations indicate that to detect a significant ($P<0.05$) difference of one sclerotium per 50 ml soil between replicate determinations a minimum of 12 determinations must be performed; as the error permitted increases, the number of replications needed declines sharply in a hyperbolic fashion.

Field studies.—Soil from plots under peanuts during the 1971 season and corn in the 1972 season did not contain sclerotia in numbers detectable by this method. Sclerotia in significant numbers (3.3/250 ml soil) were obtained from plots with corn in 1971 and peanuts in 1972.

The distribution of sclerotia in two adjacent tiers of 26 plots each is graphically represented in Fig. 4-A. Sclerotia counts varied from 0 to 19 per 250 ml of soil with an average of 3.8 and a SD of 3.6. The distribution in the field was not uniform. Numbers of sclerotia tended to be higher in some plots, with other areas in the field yielding few or no sclerotia. When data from one tier was compared with corresponding results from the other tier in this field, a highly significant positive correlation coefficient ($r = +0.61^{**}$) was obtained between the two sets of data.

Distribution of sclerotia in peanut soil from plots within two tiers separated by a 6-m-wide alley is shown in Fig. 4-B. The concn of sclerotia in this field ranged from 0 to 15/250 ml of soil with an average concn of 3.9 and a SD of 3.1. Again, sclerotia in this field were more concd in some plots; other areas in the tiers having very low concns. The linear correlation coefficient obtained from relating data from the two tiers was not significant ($r = +0.06$). The average germination of sclerotia collected in the field was 96%.

DISCUSSION.—Results of the study relating specific gravity of extracting solutions and the number of laboratory-grown sclerotia removed from soil, indicated that the use of solutions with sp gr values greater than that of water is essential for the efficient extraction of sclerotia by one-step flotation-sieving methods. Blackstrap molasses is generally available in areas with a sugarcane industry and in areas where it is used in the preparation of cattle feeds. The use of this material is not peremptory; it is possible that other inexpensive ingredients could be used to adjust the sp gr of the extracting solution. Differences in viscosity of materials selected could be a problem where these are large since high viscosity of the extracting solution may interfere with rapid settling of flocculated soil particles. If a more viscous material was used, the settling time may have to be increased.

Studies on the relation between sp gr of the extracting solution and flotation of field sclerotia could not be performed because of a lack of sufficient material. However, the high degree of recovery obtained with field sclerotia added to soil suggests that such sclerotia do not differ significantly from laboratory-produced sclerotia in their response to changes in sp gr.

No effect of the flocculating agent on germination of sclerotia exposed for the short time and at the low concn used in the method was noted. The mesh size chosen for the sieve is smaller than the finest sieve (40-mesh) used by Leach (7) in his washing procedure for extraction of sclerotia from sugarbeet fields. There is great variation in size of sclerotia according to origin, biotype, nutritional condition, and other factors (1), the finer mesh was found necessary to cover the range in size of sclerotia found in peanut fields in our area.

The use of a selective medium (2) for determining viability of sclerotia is considered essential. Leach (7), and Curl and Hansen (4) found that field sclerotia frequently have internal contaminants that interfere with germination. Therefore, it follows, that plating sclerotia on a nonselective medium may obscure viability estimation due to the antagonistic activities of accompanying microorganisms. The halo effect around viable sclerotia obtained with iodine solution on oxalate-gallic acid medium is interpreted to result from differences in retention of I_2 between the surrounding agar, and agar plus materials exuded by living sclerotia during the process of germination.

Results of experiments to study the effects of the final volume in the mixing beaker with constant soil sample size (50 ml) indicated that measurement of the 350 ml finally adopted as standard need not be very accurate. Such flexibility is important for the fast and comfortable operation of the method. However, changes in size of soil sample, without a corresponding adjustment of the volume of extracting solution used, reduced the accuracy of the method. Sample size can be increased if the amount of extracting solution is also increased (Rodriguez-Kabana et al., *unpublished*).

The variation between replicates encountered, accentuates the difficulty of obtaining homogenous distribution of sclerotia in samples, even following thorough mixing. This agrees with results obtained by Leach (7) who found the need for four replications per sample when using the washing-sieving technique. The variation between replicates cannot be attributed to the extraction procedure since the rate of recovery of sclerotia added to soil was relatively high. In using the method, the number of replicates needed per sample is determined by the degree of sensitivity required. In our studies, we concluded that five replications were adequate. The amount of time required per determination, however, is small; consequently, the number of replications per sample could easily be increased.

Comparisons of sclerotia content of soils from fields under a corn-winter fallow-peanut rotation indicate that

a single year of corn significantly reduced the number of sclerotia in Alabama. This perhaps explains the frequently reported (1, 5, 6) beneficial effect of the corn rotation in reducing "white mold" infection in peanuts.

Results of the studies on distribution of sclerotia in fields under peanut monoculture, indicate that sclerotia in the two fields were in considerably lower numbers than those found in sugarbeet fields by Leach (7). The distribution of sclerotia in the two fields emphasize that in contiguous plots, a pronounced "row effect" existed. This is suggested by the highly significant correlation coefficient obtained between data for these plots. Conversely, when an uncropped alley separated plots the coefficient was nonsignificant. The general distribution of sclerotia encountered in the two fields, although indicating areas of concn also pointed out that sclerotia were generally present everywhere. As observed by others (1), the centers of concn of sclerotia (or disease) vary according to the field; the reasons for this are not understood.

In conclusion, the method described in this paper is fast; two workers can process one sample per min. It is economical, since it uses inexpensive materials, and it has sufficient flexibility to permit the use of relatively untrained personnel. The limited field studies reported in this paper show the applicability of this method to studies of sclerotia ecology. It should be possible to extend or modify this procedure for the extraction of sclerotia of other pathogens from soil.

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